

# Epstein–Barr virus and virus human protein interaction maps

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Contributed by Elliott Kieff, March 14, 2007 (sent for review January 17, 2007)

**A comprehensive mapping of interactions among Epstein–Barr virus (EBV) proteins and interactions of EBV proteins with human proteins should provide specific hypotheses and a broad perspective on EBV strategies for replication and persistence. Interactions of EBV proteins with each other and with human proteins were assessed by using a stringent high-throughput yeast two-hybrid system. Overall, 43 interactions between EBV proteins and 173 interactions between EBV and human proteins were identified. EBV–EBV and EBV–human protein interaction, or “interactome” maps provided a framework for hypotheses of protein function. For example, LF2, an EBV protein of unknown function interacted with the EBV immediate early R transactivator (Rta) and was found to inhibit Rta transactivation. From a broader perspective, EBV genes can be divided into two evolutionary classes, “core” genes, which are conserved across all herpesviruses and subfamily specific, or “noncore” genes. Our EBV–EBV interactome map is enriched for interactions among proteins in the same evolutionary class. Furthermore, human proteins targeted by EBV proteins were enriched for highly connected or “hub” proteins and for proteins with relatively short paths to all other proteins in the human interactome network. Targeting of hubs might be an efficient mechanism for EBV reorganization of cellular processes.**

herpesvirus | interactome | replication | yeast two hybrid

Epstein–Barr virus (EBV), like all herpesviruses, infects and replicates in epithelial cells and establishes latency in specific nonepithelial cells. EBV belongs to the gamma subfamily of herpesviruses and is similar to the other human gamma subfamily herpesvirus, Kaposi sarcoma-associated herpesvirus (KSHV). Both viruses establish latency and periodically replicate in B lymphocytes. EBV has 43 “core” genes, which are common to all herpesviruses. Of the remaining 46 “noncore” genes, 6 have orthologs in beta and gamma herpesviruses, 12 are specific to the gamma subfamily, and 28 are EBV-specific. Core herpesvirus proteins are necessary for genome replication, packaging, and delivery in all cells. Some EBV-specific proteins are important in latent B lymphocyte infection, lymphoproliferative disease, Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma. The function of other EBV-specific and most gamma-specific herpesvirus proteins is less clearly understood (reviewed in refs. 1–4).

The goal of this study was to investigate interactions among EBV proteins and between EBV and human proteins in an unbiased manner. Such systematically derived protein–protein interaction or “interactome” maps could be combined with other gene-level biological information to formulate hypotheses about specific roles of EBV proteins of various functional and evolutionary classes and reveal more global properties of EBV and EBV–human interactomes. Noncore herpesvirus proteins are thought to adapt the core herpesvirus replication program to specific niches. In addition to the role of core herpesviruses

proteins in replication, core proteins may interact with noncore proteins to adapt replication to specific cell types or enable more complex pathophysiologic strategies such as modification of innate or acquired immune responses. For example, recent studies of mature EBV virions revealed they contain many gamma-specific proteins in the tegument (5). Little is known about these proteins; therefore, a more detailed mapping of their interactions with core herpesvirus proteins, other gamma-specific proteins, and human proteins is important for understanding their assembly into the tegument and their role in infection. Furthermore, although much is known about the role of some EBV-specific protein interactions with lymphocyte proteins in cell growth and survival, the effects are well delineated for relatively few proteins (reviewed in refs. 3 and 4).

Specific hypotheses about the role of EBV and human protein interactions should emerge by correlating an unbiased EBV interactome data set with available functional annotations. Indeed, interactome network maps have revealed global topological and dynamic features that relate to biological properties (6). For example, proteins with a large number of interactions, or “hubs,” in the interactome are more likely to be essential for yeast survival than proteins with a small number of interactions (7). Hub proteins can be further partitioned into those that function in specific biological modules and those that connect different modules (8). Furthermore, proteins somatically mutated in cancer tend to be interactome hubs (9). An unbiased EBV–human interactome map could be interrogated to address similar questions regarding system-level properties, which may be relevant to EBV pathogenesis and by extension, to other herpesviruses.

Systematic yeast two-hybrid (Y2H) interactome maps for viral proteins have been reported for Vaccinia virus (10) and more recently for Varicella-Zoster virus and KSHV (11). Although a preliminary viral–host interactome map has been produced for KSHV, this map was derived by experimental testing of predicted interactions and is limited by inspection bias toward specific proteins of high scientific interest in a literature-derived subset of viral–human protein interactions and by projection of

Author contributions: M.A.C. and K.V. contributed equally to this work; M.A.C., D.E.H., M.V., E.K., and E.J. designed research; M.A.C., K.V., L.X., A.M.H., A.E.E., N.L., T.H.-K., and E.J. performed research; M.A.C., K.V., M.R.C., A.V., M.V., E.K., and E.J. analyzed data; and M.A.C., K.V., D.E.H., M.V., E.K., and E.J. wrote the paper.

The authors declare no conflict of interest.

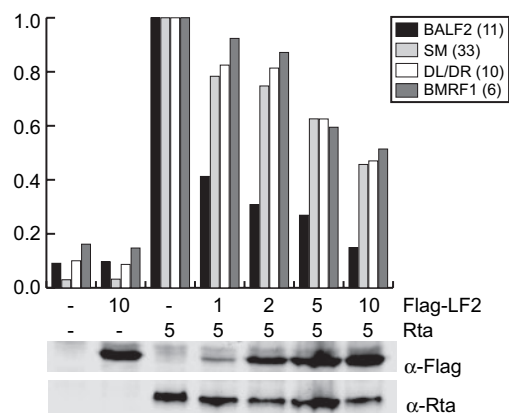
Abbreviations: Y2H, yeast two-hybrid; KSHV, Kaposi sarcoma-associated herpesvirus; DB, DNA binding; AD, activation domain; AP, affinity purification; EBNA, EBV nuclear antigen; Rta, R transactivator; ET-HP, EBV-targeted human protein; TRAF, TNF receptor-associated factor.

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This article contains supporting information online at [www.pnas.org/cgi/content/full/0702332104/DC1](http://www.pnas.org/cgi/content/full/0702332104/DC1).

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**Fig. 2.** LF2 represses Rta activation of four different EBV promoters. (Upper) BJAB cells were transfected with one of four pGL3-basic-Luc reporters containing the promoter region of an EBV gene with a well defined Rta response element (10 μg) along with 5 μg of Rta and increasing amounts (0–10 μg) of Flag-LF2. The luciferase activity shown represents the ratio of firefly luminescence over β-gal activity to normalize for transfection efficiency. The maximal response of each promoter to Rta in the absence of LF2 is shown at right (fold activation over vector only) and is normalized to 1 in the graph. The presence of LF2 significantly reduced Rta activation of all of the promoters in a dose-dependent manner. (Lower) Western blotting of cell lysates harvested 48 h posttransfection revealing that Rta expression levels were unaffected by LF2 cotransfection.

activators of immediate early gene transcription in their tegument, which promote replication in infected cells. Gamma herpesviruses may have LF2 orthologs in the tegument to impede initial replication and increase the propensity to latency in B lymphocytes.

**Global Properties of the EBV Interactome.** In addition to generating specific hypotheses of protein function, global properties of the EBV interactome were investigated. EBV proteins can be broadly divided into two evolutionary classes: herpesvirus core and noncore proteins. Of the 36 heterodimeric interactions identified in this screen, 21 (58%) were between proteins of the same evolutionary class. This statistically significant ( $P = 0.004$ ) enrichment for preferential interactions between EBV proteins of the same conservation class may have resulted from the gradual divergence of herpesvirus lineages from an ancestral strain. As gamma herpesviruses evolved, they may have gradually acquired the proteins necessary to replicate and establish latency in B lymphocytes. These proteins appear to be more likely to form complexes with one another and interact to a more limited extent with herpesvirus core proteins.

**Map of the EBV–Human Interactome Network.** Interactions between EBV and human proteins were identified by Y2H screens using 113 DB-X nonautoactivating EBV hybrid proteins against a human spleen AD-cDNA library. In total, all or part of 85 distinct EBV proteins were screened against  $10^5$  to  $10^6$  human AD-Y fusion proteins (SI Fig. 5). Positive colonies were retested by using a stringent combination of Gal4-responsive reporter genes for uracil and histidine prototrophy, β-gal activity, and 5-fluoroorotic acid sensitivity to ensure a high level of specificity. Sequencing identified 306 AD-Y in-frame interaction sequence tags and yielded 173 different EBV and human protein interactions. This EBV–human interactome map includes 40 different EBV proteins and 112 human proteins (Fig. 3 and see also SI Table 3).

**Emerging Hypotheses of Function of Specific EBV and Human Proteins.** Analyses of our map of the EBV–human interactome suggests a number of hypotheses regarding functions of EBV proteins and

their human target proteins (referred to as EBV-targeted human proteins or ET-HPs hereafter). The EBV early replication SM protein, which effects polyadenylation and cleavage of EBV DNA polymerase pre-mRNA and unspliced EBV RNA transport to the cytoplasm (18, 31, 32), interacts with the human promyelocytic leukemia nuclear body protein, Sp100, which is also known to interact with EBNA1P and mediate its coactivation of EBNA2 (33). Despite N-terminal similarity among Sp100/Sp140 family members (34), SM functionally interacts with two nonhomologous Sp110b domains (35). The interaction of SM with Sp110b is hypothesized to stabilize EBV mRNAs, and SM may interact with a nonconserved Sp100 domain for similar purposes. SM also interacted with SFRS10, an arginine/serine-rich splicing factor that is involved in regulation of splicing of mRNAs encoding cancer-associated proteins, including p53 (36) and CD44 (37). SM interaction with SFRS10 could be important in SM inhibition of human mRNA splicing. EBNA1 (amino acids 320–386) interacted with p32/TAP and EBNA1, and the herpesvirus saimiri functional equivalent, ORF73, may interact with p32/TAP to affect mRNA transport (38, 39).

Two EBV proteins required for transformation of B lymphocytes into lymphoblastoid cell lines, EBNA3A and EBNA2, interacted with several human proteins. EBNA3A interacted with two human protein regulators of apoptosis, Nur77 and RPL4. Nur77 is a nuclear hormone receptor transcription factor that can translocate to mitochondria and induce apoptosis. Interaction of Nur77 with EBNA2 localizes Nur77 to the nucleus and protects cells from Nur77-mediated apoptosis (40, 41). EBNA3A may have a similar role in preventing Nur77-mediated apoptosis. EBNA3A interaction with RPL4 may also regulate programmed cell death, as RPL4 is expressed in cells before apoptosis and forced RPL4 expression induces apoptosis (42). EBNA2 may also be targeting two pathways that modulate intracellular  $Ca^{2+}$  ion levels. EBNA2 interacts with the B cell linker protein BLNK, which is a 65-kDa Src homology 2 domain protein that interacts with Igα or LMP2A (43) and regulates intracellular  $Ca^{2+}$  (44), and with sorcin, a regulator of ryanodine receptor  $Ca^{2+}$  release (45).

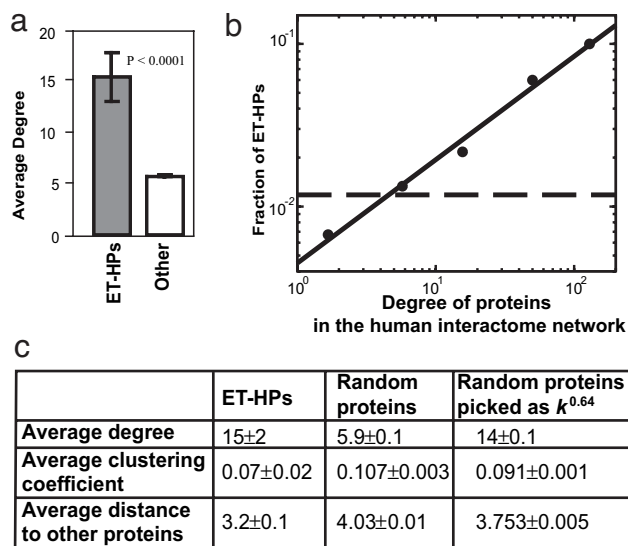
BHRF1, an EBV BCL-2 homolog expressed early in replication, interacted with the TNF receptor-associated factor 1 (TRAF1). TRAF1 is known to interact with the EBV LMP1 TRAF signaling domain (46) and has been implicated in promoting cell survival through NF-κB activation. TRAF1 interaction with BHRF1 is indicative of a direct role in cell survival during EBV replication.

**Hypotheses Regarding Human Proteins Targeted by Multiple EBV Proteins.** ET-HPs critical to the virus life cycle may be targeted by more than one EBV protein. For example, RBP-Jκ, a DB protein in the Notch signaling pathway, is targeted by four EBV nuclear proteins and three of these interactions are known to be critical for EBV-mediated B lymphocyte growth transformation (47–49). Twenty-four ET-HPs interacted with more than one EBV bait protein, accounting for 85 of 173 interactions. The high stringency used in these screens makes promiscuous binding unlikely, and highly connected protein hubs detected in other protein interaction networks analyzed to date can be biologically significant (50). For example, HSP90 interacts with a broad range of proteins and is important for specific processes such as NF-κB signaling (51).

Examination of the 13 ET-HPs with three or more Y2H interactions with EBV proteins revealed several interesting patterns. Human HOMER3 interacted with eight EBV baits, seven of which are transmembrane proteins. The apparent selectivity of HOMER3 for transmembrane proteins cannot be attributed to an ability to bind to hydrophobic transmembrane helices, as these were deleted in the EBV baits that retrieved HOMER3. Human HOMER3 localizes primarily to the endo-







**Fig. 4.** Systematic analysis of the topology and functional characteristics of ET-HPs. (a) Bar graph indicating the degree of ET-HPs in the human interactome as compared with the degree of other human proteins picked at random from the human interactome. (b) The circles represent the fraction of human proteins with degree  $k$  that are ET-HPs. The solid black line represents the best fit to  $Ak^b$ , resulting in  $b = 0.64 \pm 0.1$ . The dashed line represents the expected probability that a human protein selected at random is an ET-HP. (c) Various topological parameters of ET-HPs in the human interactome network compared with other human proteins picked randomly with uniform probability or with a probability proportional to  $k^{0.64}$ , where  $k$  is the degree of a protein in the network, are indicated.

To investigate the robustness of this correlation, we examined the average degree of both ET-HPs and random proteins that were present in various nonoverlapping subsets of the existing human interactome generated by different groups in two large-scale interactome maps (15, 55), each generated by using different Y2H technologies, as well as a data set of literature-curated interactions collated as described above. All three data sets are subject to technological biases of the assays used to detect specific interactions. Furthermore, the literature-curated data set is likely to be subject to inspection biases toward extensively studied proteins of high scientific interest. Therefore, these three data sets have different biases for detecting interactions involving particular proteins and overlap only partially in terms of protein coverage and interactions (ref. 15 and unpublished observations). Despite these biases and differences in coverage, we find that the average degree of ET-HPs present in each data set is significantly higher than the degree of other proteins. Using the Rual *et al.* (15) network, the average degree of ET-HPs is  $15 \pm 4.48$ , whereas the average degree of other proteins is  $3.2 \pm 0.16$ . In the Stelzl *et al.* (55) network, the average degree of ET-HPs is  $8.38 \pm 2.32$ , whereas the average degree of other proteins is  $3.64 \pm 0.17$ . With the literature-curated network, the average degree of ET-HPs is  $9.74 \pm 1.45$ , whereas the average degree of other proteins is  $5.44 \pm 0.11$ . This finding indicates that the preferential targeting of hubs we observed here is likely to be independent of the technical manner in which interactions were derived.

To assess the local connectivity of ET-HPs in the human interactome, we computed the average clustering coefficient, which represents the fraction of possible interactions among interactors of a given protein. The average clustering coefficient of ET-HPs is slightly smaller than that of human proteins selected at random (Fig. 4c). Because the degree of a protein in a power-law network such as the human interactome is inversely correlated with its clustering coefficient (62), we examined whether this decrease in

clustering coefficient of ET-HPs is a consequence of degree bias. We also computed as a control the average clustering coefficient for human proteins selected with a probability proportional to  $k^{0.64}$ . This choice represents a reference distribution of proteins that maintains the same overall degree distribution as that of the ET-HPs. The clustering coefficient of these control proteins was close to that of ET-HPs (Fig. 4c), which indicates that the decrease in clustering coefficient of ET-HPs compared with randomly picked human proteins follows from their degree bias.

To evaluate the extent to which proteins are “centrally” located, we considered the minimum number of interactions required to connect any “probe” human protein to any other reachable protein in the network, i.e., the “distance” to any protein present in the largest connected component. When the probe protein is an ET-HP, this average distance is smaller than when the probe is a human protein selected at random (Fig. 4c). The average distance of a protein to any other protein is inversely correlated with its degree in a power-law network such as the human interactome network. However, the shorter distance to other proteins from ET-HPs cannot be completely explained by the bias of ET-HPs toward higher-degree proteins. This assertion is supported by the fact that the average distance from ET-HPs is still smaller than the average distance from proteins selected randomly while maintaining the same overall degree distribution as ET-HPs (i.e., proteins selected randomly with a probability proportional to  $k^{0.64}$ ) (Fig. 4c). Thus, it appears that EBV proteins favor the targeting of hubs in the human interactome, and moreover, exhibit a bias toward more centrally located proteins in the human network.

## Conclusion

In summary, we have undertaken an unbiased, systematic, proteome-scale mapping of EBV–EBV and EBV–human direct protein–protein interactions. Such maps represent a rich source of protein function hypotheses, which we illustrated by demonstrating that LF2 inhibits the critical immediate early replication protein Rta. This interaction may enable the efficient establishment of latent EBV infection. Importantly, we observed a preference for interactions between EBV proteins belonging to the same evolutionary class. Further, human proteins potentially targeted by EBV tend to be hubs in the human interactome, consistent with the hypothesis that hub protein targeting is an efficient mechanism to convert pathways to virus use. The same biological properties that result in proteins being hubs in the human interactome may also result in these proteins being preferentially targeted by EBV. Finally, ET-HPs have many different functions in diverse biological pathways, consistent with the breadth of cellular machinery targeted by the virus. Although our observations are derived from incomplete sampling of the EBV and EBV–human networks, they form an important basis for comparisons to similarly sampled networks from other organisms to investigate similarities and differences. This partial understanding of the network can guide further analyses of the expanded network. Ultimately, information gained from this and other virus and viral–human interactome mapping efforts may provide an important foundation to better understand the overall organization of both viral and host proteomes and the complex interplay between their molecular machinery.

## Materials and Methods

The EBV and EBV–human interactome data sets were generated by using a high-throughput Y2H system. An EBV ORFeome, comprising 187 unique clones representing 85 of 89 EBV ORFs, was transferred from entry clones to both DB and AD vectors by Gateway recombinational cloning (12) (Invitrogen, Carlsbad, CA). The resulting constructs were transformed into haploid yeast cells. To assay EBV–EBV protein interactions the DB- and AD-transformed yeast were mated and assayed on selective media for their ability to grow in an interaction-dependent manner. The

identity of interactors was determined by PCR amplification and sequencing. For the EBV–human interactions haploid yeast containing EBV DB clones were transformed with a spleen cDNA library and selected as described above. Further details are provided in *SI Text*.

We thank F. Roth, E. Cahir-McFarland, D. Portal, and G. Szabo for helpful discussions; M. E. Cusick and F. Roth for critical reading and

editing of the manuscript; C. McCowan, C. You, C. Brennan, A. Bird, T. Clingsmith, and O. Henry-Saturne for superb administrative assistance; and C. Fraughton for laboratory assistance. This work was supported by the High-Tech Fund of the Dana-Farber Cancer Institute (S. Korsmeyer), the Ellison Foundation (M.V.), the Keck Foundation (M.V.), the National Center Institute (M.V.), the National Human Genome Research Institute (M.V.), the National Institute of General Medical Sciences (M.V.), and National Cancer Institute Grants R01CA47006 and R01CA85180 (to E.K.).

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